Ile-Glu-Asp-Lys([ε-γ]-Glu-[α-γ]-Glu)-$CONH_2$ (SEQ ID NO: 8)

and

NH_2-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Gln-Gln-$CONH_2$ (SEQ ID NO: 12).

REMARKS

Applicants submit this amendment to insert the required references to SEQ ID NOS of the Sequence Listing filed concurrently herewith into the claims, to indicate the insertion point for the Sequence Listing and to correct typographical errors. In responding to the outstanding office action dated April 22, 2002, no new matter has been added.

The examiner has also restricted the claims into the following two groups:

Group I, claims 1-9, drawn to a procytotoxin;

Group II, claims 10-19, drawn to a method of killing cells with cytotoxic peptides

With respect to the restriction between Groups I (claims 1-9) and II (claims 10-19), applicants respectfully submit that the pending claims of these two groups do not require restriction because examination of these claims would not require additional searches or otherwise place a serious burden on the examiner.

Applicants respectfully request that the examiner reconsider her position regarding this restriction requirement and examine the claims of Group I and II, as one invention for the reason set forth above. It is believed that the alleged separate inventions are related and should be examined as one invention. In addition, rejoinder may be required under the Ochiai guidelines upon a finding that the elected product is allowable.

If the examiner maintains the present restriction requirement, applicants elect, with traverse, Group I, claims 1-9.

The examiner has also required applicants to elect a single species for examination. Accordingly, applicants elect amoebapore as the species.

To the extent that claims 5 or 7 read on the elected species, the examiner has further required applicants to elect and define a single "R" species at each "R" position. Applicants therefore elect [epsilon-gamma]-Glu-[alpha-gamma]-Glu as the "R" species and elect this "R" species for the last "R" position. In the first two "R" positions, the "R" will be the unmodified ε-amino group of the lysine residue. In other words, to the extent that claim 5 reads on amoebapore, applicants elect the species represented by SEQ ID No. 8.

In regard to requiring the election of species, the examiner is reminded that upon the allowance of a generic claim, applicants are entitled to consideration of claims to additional species which are written in dependent form or contain all of the limitations of an allowed generic claim as provided by 37 C.F.R. §1.141.

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Respectfully submitted,

Date 20 June 2002

By _____

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No.19-0741for any such fees; and applicant(s) hereby petition for any needed extension of time.

MARKED UP VERSION ATTACHED TO AMENDMENT IN

SERIAL NO. 09/851,422

Marked up version of paragraph [0008], on pages 4 & 5, is below:

[0008] Particularly preferred procytotoxins have the following structures: (1) Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys(R)-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys(R)-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys(R) (SEQ ID NO: 1), and (2) Gly-Ile-Gly-Ala-Val-Leu-Lys(R)-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys(R)-Arg-Lys(R)-Arg-Gln-Gln (SEQ ID NO: 2), wherein R is independently selected from the group consisting of the unmodified ε-amino group of the adjacent lysine residue, [ε-γ]-Glu, [ε-γ]-Glu-[α-γ]-$(Glu)_{1-3}$, [ε-α]-$(Phe)_{1-3}$, [ε-α]-$(Tyr)_{1-3}$, [ε-α]-$(Trp)_{1-3}$, [ε-α]-$(Lys)_{1-3}$ and [ε-α]-$(Arg)_{1-3}$, wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate, [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate, [ε-α] represents a peptide bond between the epsilon amino acid of lysine and the alpha carboxyl group of the indicated amino acid and the subscript indicates that additional numbers of the designated amino acid can be linked to the first via conventional peptide bonds.

Marked up version of paragraph [0024], on pages 8 & 9, is below:

[0024] According to a preferred embodiment of the invention, the cytolytic peptide is melittin or an analog or derivative thereof. Melittin is isolated from bee venom and is a 26 amino acid amphiphilic alpha-helix (Blondelle et al., (1991) Biochemistry 30: 4671-4678; Dempsey et al., (1991) FEBS Lett. 281: 240-244.). The amino acid sequence of melittin is shown in Table 1. Residues 1-20 are predominantly hydrophobic and residues 21 to 26 are hydrophilic and basic. Melittin has antibiotic activity, but in mammals it is lytic for leukocytes, red blood cells and a wide variety of other cells. Compounds similar to melittin, which are also within the scope of the invention, include bombolitin from bumblebee venom (17 amino acid amphiphilic alpha-helix), mastoparan from wasp venom (14 amino acid amphiphilic alpha-helix) and crabrolin from hornet venom (13 amino acid amphiphilic alpha-helix) (Argiolas, A. and Pisano, J. J., 1985, J. Biol. Chem. 260, 1437-1444.).

TABLE 1 Amino Acid Sequence of Selected Cytolytic Peptides

Amoebapore Helix 3 (*Entamoeba histolytica*)
NH_2-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-
Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys-[COOH] $CONH_2$ (SEQ ID NO: 3)

Cecropin A (*Antheria pernyi*)
NH_2-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Glu-Lys-Val-Gly-Gln-Asn-Ile-Arg-Asp-Gly-Ile-Ile-Lys-Ala-Gly- Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-Thr-Gln-Ile-Ala-Lys-COOH (SEQ ID NO: 4)

Cecropin B (*Antheria pernyi*)
NH_2-Lys-Trp-Lys-Ile-Phe-Lys-Lys-Ile-Glu-Lys-Val-Gly-Arg-Asn-Ile-Arg-Asn-Gly-Ile-Ile-Lys-Ala-Gly-Pro-Ala-Val-Ala-Val-Leu-Gly-Glu-Ala-Lys-Ala-Leu-COOH (SEQ ID NO: 5)

Cecropin D (*Antheria pernyi*)
NH_2-Trp-Asn-Pro-Phe-Lys-Glu-Leu-Glu-Lys-Val-Gly-Gln-Arg-Val-Arg-Asp-Ala-Val-Ile-Ser-Ala-Gly-Pro-Ala-Val-Ala-Thr-Val-Ala-Gln-Ala-Thr-Ala-Leu-Ala-Lys-COOH (SEQ ID NO: 6)

Melittin (*Apis mellifera*)
NH_2-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-COOH (SEQ ID NO: 7)

Marked up version of paragraph [0034], on page 13, is below:

[0034] A particularly preferred cytotoxin is an amoebapore derivative: NH_2-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys-[COOH] $CONH_2$ (SEQ ID NO: 3).

Marked up version of paragraph [0040], on page 15, is below:

[0040] Particularly preferred procytotoxins include amoebapore, its analogs and its derivatives that contains one or more γ-linked glutamate residues linked via a peptide bond to the epsilon amino group of at least one lysine, preferably the C-terminal-most lysine (hereinafter "γ-glutamate-masked amoebapore analog"). A particularly preferred procytotoxin has the following structure: Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys-[ε-γ]-Glu-[α-γ]-(Glu) (SEQ

ID NO: 8), wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate and [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate.

Marked up version of paragraph [0041], on page 15, is below:

[0041] In addition, amoebopore and other cytotoxic peptides can be modified with other amino acids. One such exemplary protoxin has the following structure: Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys-[ε-α]-Phe (SEQ ID NO: 9), wherein [ε-α] represents a peptide bond between the epsilon amino group of lysine and the alpha carboxyl group of the adjacent phenylalanine. Another exemplary protoxin that can be activated by chymotrypsin-like activity has the following structure: Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-([ε-α]-Phe)-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys-[ε-α]-Phe (SEQ ID NO: 10), using the same nomenclature and where Lys ([ε-α]-Phe)-Leu represents a linkage between the epsilon amino group of lysine and the alpha carboxy group of phenylalanine, and a standard peptide linkage between lysine and phenlyalanine. Of course, the phenylalanine may be replaced with other amino acids, such as tyrosine and tryptophan in the case of chymotrypsin-like activity. In some instances, in order to invoke trypsin-like activity, it may be beneficial to utilize positively charged amino acids, like arginine and lysine, instead of phenylalanine.

Marked up version of paragraph [0042], on page 16, is below:

[0042] Other particularly preferred procytotoxins include melittin, its analogs and its derivatives that contain at least one γ-linked glutamate residue linked via a peptide bond to the epsilon amino group of a lysine (hereinafter "γ-glutamate-masked melittin analog"). As indicated in Table 1, melittin has two lysines and two adjacent arginines near its C-terminus. When one of the lysines is so masked, it is expected that the free alpha carboxyl group would act to neutralize the adjacent arginine, further contributing to the inhibition of the toxic activity of melittin. A particularly preferred procytotoxin has the following structure: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys([ε-γ]-Glu)-Arg-Lys([ε-γ]-Glu)-Arg-Gln-Gln (SEQ ID

NO: 11), wherein -Lys-([ε-γ]-Glu)-Arg- represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate and a standard peptide bond between the lysine and arginine residues. Of course, -Lys-([ε-γ]-Glu)-Arg- can be replaced, for example, by -Lys([ε-α]-Phe)-Leu-, as detailed above, and phenylalanine can be replaced by other amino acids like tyrosine and tryptophan to invoke chymotrypsin-like activity. In some instances, when trypsin-like activity is being invoked, it may be beneficial to utilize positively charged amino acids, like arginine and lysine, instead of phenylalanine in this latter example.

Marked up version of paragraph [0044], on page 17, is below:

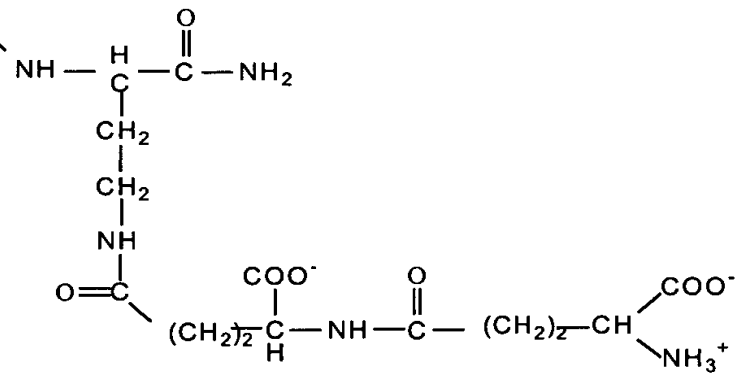
[0044] In sum, a set of particularly preferred procytotoxins have the following structures: (1) Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys(R)-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys(R)-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys(R) (SEQ ID NO: 1), and (2) Gly- Ile-Gly-Ala-Val-Leu-Lys(R)-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys(R)-Arg-Lys(R)-Arg-Gln-Gln (SEQ ID NO: 2), wherein R is independently selected from the group consisting of the ε-amino group of the adjacent lysine residue, [ε-γ]-Glu, [ε-γ]-Glu-[α-γ]-$(Glu)_{1-3}$, [ε-α]-$(Phe)_{1-3}$, [ε-α]-$(Tyr)_{1-3}$, [ε-α]-$(Trp)_{1-3}$, [ε-α]-$(Lys)_{1-3}$ and [ε-α]-$(Arg)_{1-3}$, wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate, [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate, [ε-α] represents a peptide bond between the epsilon amino acid of lysine and the alpha carboxyl group of the indicated amino acid and the subscript indicates that additional numbers of the designated amino acid can be linked to the first via conventional peptide bonds. With regard to the subscripted numbers, it is understood that larger numbers of amino acids are possible, *e.g.*, 4, 5, 6, *etc.*, but 1, 2, and 3 are anticipated to be optimal.

Marked up version of paragraph [0071], on pages 26 & 27 is below:

[0071] N-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys-[COOH] $CONH_2$ (SEQ ID NO: 3).

Procytolytic Peptide:

N-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp--



(SEQ ID NO: 8)

Example 2: Assay for the cytolytic activity of the pore-forming toxins

Marked up version of paragraph [0076], on page 28, is below:

[0076] This example demonstrates that the inventive γ-glutamate-masked cytolytic peptides have specificity for cancer cells other than those expressing PSMA. This experiment, utilized a melittin analog having A [ε-γ]-Glu-[α-γ]-Glu at each of lysines 21 and 23: NH_2-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Gln-Gln-COOH (SEQ ID NO: 12). Two prostate tumors (PNCap and DU0145), two ovarian tumors (HeLa and SK-OV-3), one lung tumor (LLC1) and one melanoma (B16) were tested. Cultured cells were treated with 1, 10, 50 or 100 μM peptide. Results, depicted in Figure 4, show strong lytic activity against all tumors.

In the Claims:

Please amend the claims as follows:

5. (Amended) The procytotoxin of claim 4, having the following structure: Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys(R)-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys(R)-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys(R) (SEQ ID NO: 1), wherein R is independently selected from the group consisting of the unmodified ε-amino group of the adjacent lysine residue, [ε-γ]-Glu, [ε-γ]-

Glu-[α-γ]-$(Glu)_{1-3}$, [ε-α]-$(Phe)_{1-3}$, [ε-α]-$(Tyr)_{1-3}$, [ε-α]-$(Trp)_{1-3}$, [ε-α]-$(Lys)_{1-3}$ and [ε-α]-$(Arg)_{1-3}$, wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate, [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate, [ε-α] represents a peptide bond between the epsilon amino acid of lysine and the alpha carboxyl group of the indicated amino acid and the subscript indicates that additional numbers of the designated amino acid can be linked to the first via conventional peptide bonds.

7. (Amended) The procytotoxin of claim 6, having the following structure: Gly-Ile-Gly-Ala-Val-Leu-Lys(R)-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys(R)-Arg-Lys(R)-Arg-Gln-Gln (SEQ ID NO: 2), wherein R is independently selected from the group consisting of the unmodified ε-amino group of the adjacent lysine residue, [ε-γ]-Glu, [ε-γ]-Glu-[α-γ]-$(Glu)_{1-3}$, [ε-α]-$(Phe)_{1-3}$, [ε-α]-$(Tyr)_{1-3}$, [ε-α]-$(Trp)_{1-3}$, [ε-α]-$(Lys)_{1-3}$ and [ε-α]-$(Arg)_{1-3}$, wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate, [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate, [ε-α] represents a peptide bond between the epsilon amino group of lysine and the alpha carboxyl group of the indicated amino acid and the subscript indicates that additional numbers of the designated amino acid can be linked to the first via conventional peptide bonds.

8. (Amended) The procytotoxin of claim 1 having a structure selected from the group consisting of:

[N-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Ile-Gln-Leu-Ile-Glu-Asp-Lys([ε-γ]-Glu-[α-γ]-Glu)-COOH] N-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys([ε-γ]-Glu-[α-γ]-Glu)-$CONH_2$ (SEQ ID NO: 8)

and

NH_2-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Gln-Gln-COOH (SEQ ID NO: 12).

16. (Amended) The method of claim 14, wherein the procytotoxin has the following structure: Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys(R)-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys(R)-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys(R) (SEQ ID NO: 1), wherein R is independently selected from the group consisting of the unmodified ε-amino group of the adjacent lysine residue, [ε-γ]-Glu, [ε-γ]-Glu-[α-γ]-$(Glu)_{1-3}$, [ε-α]-$(Phe)_{1-3}$, [ε-α]-$(Tyr)_{1-3}$, [ε-α]-$(Trp)_{1-3}$, [ε-α]-$(Lys)_{1-3}$ and [ε-α]-$(Arg)_{1-3}$, wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate, [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate, [ε-α] represents a peptide bond between the epsilon amino acid of lysine and the alpha carboxyl group of the indicated amino acid and the subscript indicates that additional numbers of the designated amino acid can be linked to the first via conventional peptide bonds.

18. (Amended) The method of claim 17, wherein the procytotoxin has the following structure: Gly- Ile-Gly-Ala-Val-Leu-Lys(R)-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys(R)-Arg-Lys(R)-Arg-Gln-Gln (SEQ ID NO: 2), wherein R is independently selected from the group consisting of the unmodified ε-amino group of the adjacent lysine residue, [ε-γ]-Glu, [ε-γ]-Glu-[α-γ]-$(Glu)_{1-3}$, [ε-α]-$(Phe)_{1-3}$, [ε-α]-$(Tyr)_{1-3}$, [ε-α]-$(Trp)_{1-3}$, [ε-α]-$(Lys)_{1-3}$ and [ε-α]-$(Arg)_{1-3}$, wherein [ε-γ] represents a peptide bond between the epsilon amino group of lysine and the gamma carboxyl group of the adjacent glutamate, [α-γ] represents a peptide bond between the alpha amino group of the first glutamate and the gamma carboxyl group of the second glutamate, [ε-α] represents a peptide bond between the epsilon amino group of lysine and the alpha carboxyl group of the indicated amino acid and the subscript indicates that additional numbers of the designated amino acid can be linked to the first via conventional peptide bonds.

19. (Amended) The method of claim 17 wherein the procytotoxin has a structure selected from the group consisting of:
[N-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Ile-Gln-Leu-Ile-Glu-Asp-Lys([ε-γ]-Glu-[α-γ]-Glu)-COOH] N-Gly-Phe-Ile-Ala-Thr-Leu-Cys-Thr-Lys-Val-Leu-Asp-Phe-Gly-Ile-Asp-Lys-Leu-Ile-Gln-Leu-Ile-Glu-Asp-Lys([ε-γ]-Glu-[α-γ]-Glu)-$CONH_2$ (SEQ ID NO: 8)
and

NH_2-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Lys([ε-γ]-Glu-[α-γ]-Glu)-Arg-Gln-Gln-COOH (SEQ ID NO: 12).